DNA cloning in Bacillus subtilis
(plasmids/recombinant molecules/heterospecific gene expression)

STANISLAV D. EHRICH
Institut de Recherche en Biologie Moléculaire, Laboratoire d’Etude des Acides Nucléiques, Université Paris VII, 2 Place Jussieu, 75005 Paris, France
Communicated by Joshua Lederberg, December 23, 1977

ABSTRACT A plasmid pC194, encoding resistance to chloramphenicol, can serve as a cloning vector in Bacillus subtilis 168 for other HindIII-cleaved DNA segments. Replicons constructed by linking pC194 to several Escherichia coli plasmids can be used to introduce and compare the expression of the same genes in these two bacterial hosts.

Since the pioneering experiments of Cohen and his colleagues (1), the technology of DNA cloning in Escherichia coli has become a major tool of molecular genetics. The main extensions beyond E. coli experiments are with plasmid RP4 and its wide range of Gram-negative hosts (2).

Bacillus subtilis is a Gram-positive, sporulating, aerobic bacterium phylogenetically very distant from E. coli. A B. subtilis cloning system (3) parallel to that developed for E. coli permits studies of the interaction of the same genes with two, possibly very different, cellular environments, which would offer greater insight into the diverse processes that convert genetic information to the corresponding phenotype.

The development of the B. subtilis cloning system has been mainly hindered by the absence of suitable vector replicons. Several plasmids can be introduced and maintained in the highly transformable B. subtilis strain 168 (4, 5); however, they lacked genetic markers for selection and DNA cloning. More recently, plasmids originally isolated from Staphylococcus aureus that code for resistance to tetracycline or chloramphenicol have been transfected into B. subtilis (6). These can be tested for their ability to accept inserted DNA segments, while still maintaining the capacity to replicate and express their genetic information in this host. The present experiments show that DNA segments can be inserted into the chloramphenicol resistance plasmid pC194, which then constitutes a DNA cloning vector for B. subtilis 168.

MATERIALS AND METHODS

Bacterial Strains. E. coli strains used were W5444 thr-1 leu-6 thi-1 supE44 lacY1 r- m- strB (tonB trpD trpE trpF trpG trpH trpI trpL trpM trpN trpO trpP trpQ trpR trpS trpT trpU trpV trpW trpX trpY trpZ) (delta). The following plasmids were carried in the C600 strain: pSC105 (1), pBR322 (7), pBR313 (8), pMB9 (8), and pW17 (from W. Goebel). B. subtilis strains included SB202 trpC2 trpE2 trpF2 trpG2 trpH2 trpI2 trpJ2 trpK2 trpL2 trpM2 trpN2 trpO2 trpP2 trpQ2 trpR2 trpS2 trpT2 trpU2 trpV2 trpW2 trpX2 trpY2 trpZ2 (tonB trpD trpE trpF trpG trpH trpI trpJ trpK trpL trpM trpN trpO trpP trpQ trpR trpS trpT trpU trpV trpW trpX trpY trpZ) (delta). E. coli-B. subtilis hybrid replicons were constructed by linking pC194 to several plasmids from E. coli and B. subtilis.

DNAs and Enzymes. Plasmid DNAs were prepared by the cleared lysis procedure (9). The HindIII, BamHI, and Psi restriction endonucleases were commercial preparations (Biolabs, Beverly, MA). Hae III was a gift from V. Saramella. The EcoRI endonuclease and the T4 DNA ligase were isolated and used as described (10, 11).

Transformation Procedure. Competence-induction and transformation of E. coli and B. subtilis strains were done by published procedures (12, 13). Potential biohazards associated with the experiments described in this publication have been reviewed by the French National Control Committee. The appropriate experiments were performed at P2 containment level.

Electrophoresis. Endonuclease-digested DNA was separated on horizontal agarose gels essentially as described (14).

RESULTS

In vitro construction of B. subtilis plasmid resistant to tetracycline and chloramphenicol

pC194 has a single HindIII site and pT127, a B. subtilis plasmid that encodes resistance to tetracycline, carries three such sites (6). This prompted the attempt to insert the HindIII-generated segments of pT127 into the HindIII site of pC194. The results are summarized in Table 1.

As expected (6), the HindIII cleavage destroyed the transforming activity of the plasmid DNAs. This could be restored by T4 ligase treatment of cleaved pC194, but not of pT127. However, if the two DNAs were ligated together, some TcR clones could be recovered. These were resistant to chloramphenicol as well, and contained plasmid DNA (shown by ethidium bromide/CsCl density gradient centrifugation). A hybrid plasmid, designated pHV11, was characterized by electrophoresis and transformation. The results are summarized in Table 2.

The size of pHV11 equals the sum of that of pC194 and of the largest of the HindIII segments of pT127. HindIII treatment of pHV11 DNA gives rise to two DNA segments, which match the HindIII-cleaved pC194 and the slowest of the pT127 segments in electrophoretic mobility (Fig. 1). pHV11 plasmid DNA transforms B. subtilis, conferring resistance to tetracycline and chloramphenicol. These data are consistent with the structure shown in Fig. 2: the 1.5 x 10^6 dalton DNA segment of pT127, carrying the TcR marker, has been inserted into the HindIII site of pC194.

Construction of E. coli-B. subtilis hybrid replicons

The results presented in the preceding section indicate that pC194 can be used as a HindIII cloning vector in B. subtilis. The insertion of DNA segments other than those of the plasmid pT127 into pC194 should therefore be feasible. This was tested by constructing hybrid replicons between pC194 and several E. coli plasmids, each containing a single HindIII cleavage site. For this purpose, pBR313, pBR322 (both carrying ApR TcR (8, 7)), or pW17 (coding for ApR KmR, W. Goebel, personal communication) were cleaved with HindIII and ligated to HindIII-treated pC194, and the hybrid DNAs were used to

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Abbreviations: Resistance to: ApR, ampicillin; CmR, chloramphenicol; KmR, kanamycin; TcR, tetracycline; TnA, transposable element coding for resistance to ampicillin.
B. subtilis cell suspension (0.1 ml), at the competence level of 0.2%, was mixed with 0.2 μg of the corresponding DNA, incubated for 30 min, and plated on L plates supplemented with 3 μg of chloramphenicol or 15 μg of tetracycline per ml. HindIII cleavage was complete, and the ligase reaction was about 50% complete, as judged by agarose gel electrophoresis.

Many colonies that displayed the expected marker inactivation and also acquired resistance (to 100 μg/ml) of chloramphenicol were isolated. Plasmid DNA was extracted from representative clones and analyzed by electrophoretic and genetic tests. The results are summarized in Table 3.

In all cases the hybrid plasmids isolated from E. coli were of a size equivalent to the sum of the parents (i.e., the E. coli plasmid and pC194), and were cleaved by HindIII endonuclease into segments having mobilities that matched those of the cleaved parental DNAs (Fig. 1). These results show that hybrids between E. coli plasmids and pC194 were successfully constructed. Their structures are represented schematically in Fig. 2.

In all cases the hybrid DNAs could transform E. coli not only to ampicillin resistance, but also to chloramphenicol resistance. This confirms the observed phenotype of the colonies selected originally (see above) and represents another example of functional, heterospecific gene transfer among prokaryotes. The plasmids pHV12, pHV14, and pHV15 transformed E. coli for both markers with essentially the same efficiency. pHV16, however, transformed 1/1000 less efficiently for chloramphenicol resistance. This was due not to the absence of the gene encoding the resistance in a fraction of the plasmid population, since the 100 ApR clones tested by replica-plating were CmR as well. It may possibly be due to inefficient expression of the chloramphenicol resistance gene in pHV16 under the transformation conditions used.

In all cases transformation of B. subtilis with the hybrid DNAs gave rise to chloramphenicol-resistant transformants (Fig. 3). The number of these was proportional to the amount of pHV12 or pHV14 DNA, up to about 10 ng. The efficiencies were close to 10^9 transformants per μg of DNA, 30–100 times higher than the value observed with pC194 or pHV11 DNAs. pHV16 DNA transformed much less efficiently, and no proportionality between the number of transformants and the amount of DNA was observed.

In all cases the B. subtilis CmR transformants have acquired

![Fig. 1. Electrophoretic analysis of HindIII-cleaved B. subtilis plasmids. Cleaved DNA (0.1–0.2 μg) was analyzed on a 0.5% horizontal agarose gel. Lanes a–i contain HindIII-cleaved DNAs: (a) pC194; (b) pT127; (c) pHV11; (d) pBR322; (e) pHV12; (f) pHV14; (g) pHV14; (h) pWL7; and (i) pHV16. Lane j contains EcoRI-cleaved Phi-3-T DNA size marker (10).](image)

![Fig. 2. Schematic representation of several hybrid plasmids isolated from B. subtilis. pHV15 differs from pHV14 with respect to the relative orientation of pC194 and pBR322 DNA segments, as determined by comparing the Hae III restriction patterns of the two plasmids.](image)
plasmids matching the plasmids isolated from E. coli (not shown) in size and HindIII restriction pattern. This confirms that pC194 is able to serve as a vector for cloning HindIII-generated DNA segments in B. subtilis.

The hybrid plasmid pHV14 contains unique sites for EcoRI, Bam, and Pst, pHV12 for EcoRI and Bam. All of these are located within the part of the hybrid genomes derived from E. coli plasmids. Insertion of DNA segments at any of these sites does not interfere with the replication of the parental plasmids in E. coli. It is therefore very likely that pHV14 and pHV12 can be used as cloning vectors for DNA segments produced with these restriction enzymes in both E. coli and B. subtilis. This was confirmed for an EcoRI-generated segment introduced in pHV12 (see below).

Lack of expression of E. coli genes in B. subtilis

The hybrid plasmids described in the preceding section carry the gene specifying resistance to ampicillin. It is present either within the intact transposon TnA (pHV16) or as a deleted derivative of TnA (pHV12 and pHV14). This gene failed to confer resistance to ampicillin on B. subtilis. Both the parental strain and the CmR transformants that contained hybrid plasmids were inhibited by 0.5 μg of ampicillin per ml. In the same hybrid plasmid the failure to change the drug-sensitive phenotype was not due to a structural modification (e.g., deletion) within the Ap gene. The plasmid DNAs extracted from the B. subtilis CmR host transformed E. coli to both chloramphenicol and ampicillin resistance with equal efficiency (except in pWB7, see above) while they transformed B. subtilis to the chloramphenicol resistance only.

The lack of expression of the ApR gene is not related to its orientation relative to the pC194 part of the hybrid plasmid since pHV15, a plasmid in which pBR322 is inserted in the opposite orientation to that in pHV14, did not confer ampicillin resistance to B. subtilis.

These results prompted further investigation of the expression of E. coli genes in B. subtilis. For that purpose the DNA segment that carries the gene specifying resistance to kanamycin was excised from pSC105 (1) by the action of EcoRI and inserted into the EcoRI site of pHV12 by standard techniques. The resulting plasmid, pHV18, conferred resistance to ampicillin, chloramphenicol, and kanamycin on the E. coli host. It rendered B. subtilis resistant only to chloramphenicol, transforming competent cells at an efficiency essentially identical to that of the parental pHV12 DNA (about 10^6 transformants per μg of DNA). Both the CmR transformants and the parental strain were inhibited by concentrations between 0.5 and 1 μg of kanamycin per ml.

Plasmid DNA isolated from the CmR B. subtilis transformants matched the parental E. coli plasmid pHV18 in size and HindIII and EcoRI patterns. This DNA could transform E. coli to ApR, CmR, and KmR with essentially equal efficiency, demonstrating the presence of the functional KmR gene on the plasmid.

These experiments show that pHV12 can be used as an EcoRI cloning vector in the B. subtilis system and that the E. coli KmR gene is not expressed in the B. subtilis host.

**DISCUSSION**

The experiments reported here establish a cloning system in a B. subtilis host with pC194 as a vector for DNA segments by HindIII. In addition, two hybrid plasmids (pHV12 and pHV14) were constructed that can serve as cloning vehicles in both E. coli and B. subtilis for DNA segments by several restriction enzymes (EcoRI, Bam, and Pst). They can transform both hosts at equal efficiency of about 10^6 transformants per μg of DNA.

The genes carried on pHV12 or pHV14 plasmids can be introduced, and their behavior studied, in both B. subtilis and E. coli. The model system described in this paper deals with E. coli genes specifying resistance to ampicillin or kanamycin. Both confer, of course, the drug-resistance phenotype upon their natural host. However, they fail to express their information in B. subtilis for reasons still to be learned. The resistance to

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**Table 3. Construction of hybrid replicons between several E. coli plasmids and pC194**

<table>
<thead>
<tr>
<th>Plasmid (Origin*)</th>
<th>HindIII segments</th>
<th>Transforming activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR313 (A)</td>
<td>6.1</td>
<td>Tc Ap  None</td>
</tr>
<tr>
<td>pHV12 (pBR313 + pC194)</td>
<td>7.9, 6.1, 1.8</td>
<td>Cm Ap  Cm</td>
</tr>
<tr>
<td>pHV14 (pBR322 + pC194)</td>
<td>2.8, 2.8</td>
<td>Tc Ap  None</td>
</tr>
<tr>
<td>pWL7 (C)</td>
<td>4.6, 2.8, 1.8</td>
<td>Cm Ap  Cm</td>
</tr>
<tr>
<td>pHV16 (pWL7 + pC194)</td>
<td>7.9, 7.9, 1.8</td>
<td>Km Ap  None</td>
</tr>
</tbody>
</table>

E. coli W5445 cells were used as recipients in transformation, which was performed as previously (12). 10^6-10^8 transformants per μg of DNA were usually obtained. The sizes of the DNA segments were determined as described in the footnote to Table 2. Additional covalently closed circular size markers were pBR313 and pWL7, which were calibrated after HindIII cleavage, using EcoRI-treated Phi-3-T DNA as the size standard.

ampicillin appears particularly interesting, since this gene, which specifies TEM β-lactamase, has been shown to function in prokaryotes taxonomically distant from E. coli such as Haemophilus influenzae (15) and Neisseria gonorrhoeae (16).

The behavior of the two E. coli genes studied here in B. subtilis can be contrasted with that of the B. subtilis CmR gene introduced into E. coli and that of S. aureus ApR (17) and B. subtilis thy (10), leu (18), or pyr (Chi, N. Y., Ehrlich, S. D., and Lederberg, J., J. Bacteriol., in press), which are all expressed in E. coli. As a start for generalizations from these data, more examples of the behavior of E. coli genes in B. subtilis are being studied. One such case is that of tetracycline resistance. The B. subtilis genetic information, when introduced into E. coli (by inserting the 1.5-megadalton HindIII segment of pT127 into pWL7, not shown), conferred resistance upon that host, which indicates some similarity in the structure of membranes in prokaryotes as diverse as S. aureus (from which the gene was originally isolated), B. subtilis, and E. coli. The reciprocal transfer of E. coli TeR genes into B. subtilis is under study.

I am indebted to my colleagues from the Institut de la Recherche en Biologie Moléculaire for their help in creating a productive laboratory environment. I thank Mrs. E. Pierre for technical assistance, the participants of the EMBO course in Genetic Engineering, Paris 1977, who enthusiastically performed some of the experiments, and Dr. S. Jupp for help in preparing the manuscript. This research was supported, in part, by a grant from the Federation de la Recherche Medicale Francaise.